MAMMALIAN ICYP (IODOCYANOPINDOLOL) RECEPTOR AND ITS APPLICATIONS

The present invention relates to an isolated and substantially pure mammal polypeptide different from known adrenergic, serotonine and dopamine receptors, existing at least on mammalian muscle and eosinophils membranes, for instance in rat, guinea pig and humans.

The invention also relates:

to plasmids containing the genes coding for said polypeptide,

to host cells transformed by genes coding for the above mentioned polypeptide,

to nucleotide probes capable of hybridizing with the genes coding for the above mentioned polypeptide, and

to polyclonal and monoclonal antibodies directed against the above mentioned polypeptide and which can be used for the purpose of *in vitro* diagnosis,

A wide variety of membrane receptors for hormones and neurotransmitters are composed of a single polypeptide chain containing seven hydrophilic sequences and may be coupled to guanine-nucleotide-binding regulatory G proteins, which upon activation by agonists or antagonists, stimulate or inhibit various effectors such as enzymes or ion channels.

Among the family of seven transmembrane domains receptors are those for adrenaline and other catecholamines, the adrenergic receptors and those for acetylcholine and related muscarinic ligands, the muscarinic cholinergic receptors. Other similar proteins belonging to this growing family are those for serotonine, for dopamine, for tachykinins and for the pituitary glycoprotein hormones, to mention but a few.

The existence of atypical adrenergic receptors (AR), in adipocytes, in gastrointestinal tissues and in skeletal muscles has been well-established. Atypical β-adrenergic receptors (β-ARs) are defined as β-AR that can not be classified as typical β-ARs (β1-AR and β2-AR) with low β-AR antagonist effect, showing a propranolol (a classical non-selective β-AR antagonist)-resistant feature.

For instance, McLaughlin, MacDonald and co-workers characterized β-AR in rat colon (McLaughlin, MacDonald, 1990; MacDonald and Lamont, 1993; McKean and MacDonald, 1995). Propranolol was a weak antagonist against isoproterenol and BRL-37344. The propranolol-resistant responses of isoproterenol were antagonized by cyanopindolol with a pA_2 value of 7.12 under blockade of β1- and β2-AR effects. They reported that responses to isoproterenol in rat colon were mediated largely through β3-AR with small contribution of β1-AR and β2-AR (McKean and MacDonald, 1995). This observation is supported by Ek et al., 1986, who found β1- and β2-AR in rat colon membranes by [^{125}I]-pindolol binding studies. Thus, rat colon has mainly β3-AR in addition to β1- and β2-ARs. Like in guinea pig ileum, cyanopindolol acted as an antagonist at rat atypical β-AR, while it acted as a β1-, β2-AR antagonist having β3-AR agonist potency at human and mouse β3-AR (Blin et al., 1993).

Most of the pharmacological features of atypical β-ARs can be explained by β3-AR-activity; however, lack of β3-AR transcripts in skeletal muscles, or heterogeneous responses in vascular smooth muscles remained unexplained and show the complexity found in the field of receptors.

The invention solves an unresolved question with regard to the existence of polypeptide having a receptor activity other than that of β3-adrenergic receptors; in fact, it provides access to a novel receptor class present at least in muscles and in eosinophils, which displays transmembrane domains and may have signal transduction function.

The Inventors have now found, unexpectedly, that in rat colon smooth muscle membranes, there is a non-adrenergic, non-serotonine and non-dopamine receptor mediating at least inhibition of depolarized colon tonus.

The subject of the present invention is a substantially pure mammal polypeptide containing sites such that when said sites are exposed at the surface of a cell, they are able of binding iodocyanopindolol (ICYP) under blockade of α, β1, β2, β3-AR, serotonine 5-HT_{1A} and serotonine 5-HT_{1B} receptors, said binding being saturable, reversible, able to be displaced by a β-adrenergic receptor agonist SM-11044 with stereo-

selectivity but not by isoproterenol, norepinephrine, epinephrine, serotonine, dopamine or BRL-37344, and not being blocked by propanolol said polypeptide (1) having an apparent molecular weight of about 30-40 kDa when labeled with ^{125}I-iodocyanopindolol after photoaffinity labeling and separation by electrophoresis and an apparent molecular weight of about 60-80 kDa in Western blot, and (2) generating a fragment having the following formula $DPX_1FFQHRIHX_2FSIFNX_3$ by acidic cleavage, wherein, X_1 represents S (SEQ ID N°5) or X (SEQ ID N°6), X_2 represents V (SEQ ID N°6) or W (SEQ ID N°5) and X_3 represents S (SEQ ID N°5) or H (SEQ ID N°6), said polypeptide being present at least on muscles and eosinophils membranes and being a non-adrenergic receptor.

Said new non-adrenergic receptor has the following affinities with different β3-AR agonists and antagonists:

SM-11044 stimulates guinea pig ileum relaxation of KCl-induced tonus more efficiently than rat white adipocyte lipolysis; SM-11044 and BRL-35135A, a potent β3-AR agonist, display the additional property of inhibiting leukotriene B4 induced-guinea pig eosinophil chemotaxis, whereas isoproterenol and BRL-37344 had no such effect. This inhibition was unaffected by the non-selective β-AR antagonist, propranolol, but was antagonized by alprenolol, a β1-, β2-AR antagonist/β3-AR partial agonist.

While rat colon indeed contains β3-AR (Bensaid M. et al., 1993) in addition to β2-AR with a small population of β1-AR (Arunlakshana O. et al., 1959), the instant invention clearly shows the existence of a novel functional binding site in rat colon. This site was characterized by ligand binding and photoaffinity labeling, revealing a novel binding protein, designated here Ro-SMBP (SM-11044 binding protein or Rodent SM-binding protein).

Said new non-adrenergic SM-binding protein has also been found in human muscles (smooth and striated) (Hu-SMBP), it contains at least the sequence SEQ ID NO:1.

According to an advantageous embodiment of said protein it consists of SEQ ID NO:13.

Said protein contains a hydrophobic C-terminal region of 356 residues, which may contain up to nine transmembrane regions.

The invention also relates to an isolated and purified nucleic acid which encodes a mammalian receptor as hereabove defined and fragments thereof.

In humans, said coding sequence includes at least SEQ ID NO:2.

According to an advantageous embodiment of said coding sequence, it consists of SEQ ID NO:14, which corresponds to SMBP cDNA.

The said SEQ ID NO:14 comprises in particular the following single restriction sites: BstU I, Hha I, HinP I, Ava I, Sma I, Xma I, BsaA I, Apa I, Ban II, Bsp120 I, Eco0109 I, Sca I, Xmn I, Dra I, Nsi I, Ppu10 I, Acc65 I, Ban I, Kpn I, Bsp1407 I, Spe I, BspD I, Cla I, Hinf I, Tfi I, Avr II, Drd I, Esp3 I, Bpm I, PflM I, Bsm I, Alu I, BceF I, Bgl II, BstY I, ApaL I, Age I, BsrF I, Nsp I, Nsp7524 I, NspC I, as located in figures 19, 20 and 21.

This sequence encodes a polypeptide of 576 amino acid residues which contains a hydrophilic N-terminal region of 220 residues and a hydrophobic C-terminal region of 356 residues.

Said nucleic acid sequences in different mammals at least hybridizes with:

- a 900 bp of SEQ ID NO:3, or
- a 300 bp of SEQ ID NO:4.

Said fragments are useful for detection of the gene coding for the instant new non-adrenergic receptor.

The subject of the present invention is also cDNA clones, characterized in that they comprise a sequence fragment coding for the instant non-adrenergic receptor.

According to the invention, the clone designated 24.3 comprises 1,7 kb and includes SEQ ID NO:2; it encodes the instant Hu-SMBP.

The invention also relates to synthetic or non-synthetic nucleotide probes, characterized in that they hybridize with one of the nucleic acid as defined above or with their complementary sequences or their corresponding RNA, these probes being

such that they do not hybridize with the genes or the messenger RNA coding for β-adrenergic receptors.

Said probes are selected, for instance, from the group consisting of the hereabove mentioned 900 bp (SEQ ID NO:3) and 300 bp (SEQ ID NO:4) fragments and from SEQ ID NO:7 to SEQ ID NO:12, optionally labeled using a label such as a radioactive isotope, a suitable enzyme or a fluorochrome.

SEQ ID NO:7 to SEQ ID NO:12 may be used as primers for amplifying one of the instant nucleic acid sequence.

The hybridization conditions are defined as follows, for the probes possessing more than 100 nucleotides: 600 mM NaCl; 60 mM Na-citrate; 8 mM Tris-HCl pH 7,5; 50 mM Na-phosphate; 1% Ficoll; 1% polyvinylpyrrolidone; 1% bovine serum albumine; 40% formamide; 0.2% SDS; 50 µg/ml salmon sperm DNA.

The invention also relates to recombinant plasmid, cosmid or phage in particular for cloning and/or expression, containing a nucleic acid sequence of the invention at one of its cloning sites (non essential for its replication).

According to an advantageous embodiment of the said plasmid, it further comprises an origin of replication for replication in a host cell, at least one gene whose expression permits selection of said host cell transformed with said plasmid, and a regulatory sequence, including a promoter permitting expression of a polypeptide having a non-adrenergic activity as defined hereabove, in said host cell.

According to an advantageous arrangement of this embodiment, the said plasmid is pcDNA3 into which is inserted, in a multisite linker, SEQ ID NO:2, wherein said plasmid is deposited with the Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures] (CNCM held by the PASTEUR INSTITUTE, dated December 10, 1996, under No. I-1795.

The invention also relates to a host cell transformed by a recombinant plasmid as previously defined comprising the elements of regulation making possible the expression of the nucleotide sequence coding for the instant polypeptide in this host.

Such a cell is capable of expressing a SMBP according to the instant invention.

According to an advantageous embodiment, the host cell consists, in particular, in mammalian cell lines.

The invention also relates to antibodies directed specifically against the instant polypeptide, these antibodies being such that they recognize neither known α or β adrenergic, nor serotonine, nor dopamine receptors.

Advantageously, said new non-adrenergic receptor according to the invention constitute a tool for the selection of ligand participating in the activation or in the inhibition of these receptors.

The invention also relates to a method for assaying a substance for agonist or antagonist activity towards a polypeptide according to the invention, which method comprises:

- placing the substance in contact with tissue membrane proteins or a transformed host cell expressing a polypeptide according to the invention under conditions which permit binding between said polypeptide binding sites and an agonist or an antagonist thereto and

- measuring an appropriate transduction signal.

The invention also relates to a process for studying the binding affinity of a compound for a polypeptide according to the invention, which process comprises:

- transforming a host cell by an expression vector comprising a nucleotide sequence coding for the instant receptor,

- culturing said transformed host cell under conditions which permit the expression of said receptor encoded by said nucleotide sequence and the transfer of the expressed receptor polypeptide to the membrane of the said transformed host cell so that transmembrane sequences of said receptor polypeptide are embedded in the cell membranes of the transformed host cell;

- placing said transformed host cell in contact with said compound and

- measuring the quantity of said compound bond to said receptor polypeptide.

The invention also relates to a process for studying the binding affinity of a compound for a polypeptide according to the invention, which process comprises:

- extracting membrane proteins corresponding to the instant receptor polypeptide from appropriate tissues or cells such as muscles,

- placing said membrane proteins in contact with said compound and

- measuring the quantity of said compound bond to said receptor polypeptide.

Functional roles of this polypeptide receptor would involve relaxation of depolarized-intestinal smooth muscle or inhibition of eosinophil chemotaxis.

Specific agonist for this new receptor will display at least a therapeutic potentiality in gastrointestinal diseases based on depolarized-tonus, allergic asthma and hypereosinophilic syndrome based on eosinophil accumulation.

Thus, the instant polypeptide receptor makes possible to develop drugs for at least gastrointestinal diseases based on depolarized-tonus, allergic asthma and hypereosinophilic syndrome.

Besides the foregoing arrangements, the invention also comprises other arrangements which will become apparent from the description which follows, reference being made to the attached drawings wherein:

. Figure 1: Preparative SDS-PAGE followed by autoradiography of 50 mg solubilized rat colon membranes photoaffinity-labeled with 0.5nM [^{125}I]-ICYP-diazirine in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol;

. Figure 2: Analytical chemical cleavage of SMBP. The isolated-labeled protein of 34 kDa was incubated with distilled water (lane 1), 70% formic acid (lane 2), 10% cyanogen bromide in 70% formic acid (lane 3), 75% trifluoroacetic acid (lane 4) or 10% cyanogen bromide in 75% trifluoroacetic acid (lane 5) for 24 h at room temperature, separated by Tricine-SDS-PAGE followed by autoradiography. Arrows show 8, 10 and 12 kDa labeled fragments;

. Figure 3: Preparative cyanogen bromide-cleavage of SMBP. The isolated-labeled proteins of 34 kDa were incubated with 10% cyanogen bromide in 70% formic acid for 24 h at room temperature. An aliquot of the cleaved-products was resolved on tricine-SDS-PAGE followed by autoradiography. Arrows show 8, 10 and 12 kDa labeled fragments.

. Figure 4: Analytical chemical cleavage of SMBP. Fig. 4a: the partially purified labeled proteins were incubated with distilled water (lane 1), 70% formic acid (lane 2) or 1% cyanogen bromide in 70 % formic acid (lane 3) for 24 h at room temperature or Fig. 4b: the partially purified labeled proteins were incubated with distilled water (lane 1), 70% formic acid (lane 2) for 72 h at 37°C, separated by Tricine-SDS-PAGE and subjected to autoradiography.

. Figure 5: Preparative acid-cleavage of SMBP. The isolated-labeled protein of 34 kDa was incubated with 70% formic acid for 72 h at 37°C. An aliquot of the cleaved-products was resolved on tricine-SDS-PAGE followed by autoradiography. Attows shows 8 kDa labeled fragment.

. Figure 6: Reverse-phase HPLC purification of the photoaffinity-labeled formic acid-cleaved 8 kDa fragment. The fragment isolated from tricine-SDS-PAGE gels was further purified by reverse-phase HPLC. Fragment was eluted from the C4 column with a linear gradient of 30-98% buffer B in 120 min (----). Radioactive profile for 8 kDa labeled fragment was shown (●). Based on the amount of recovered radioactivity, HPLC column recovery was 91.6%.

. Figure 7: Enzyme immunoassay (ELISA) of antiserum (●), preimmunized-serum (○) or affinity-purified antibody (■, α8-antibody) on plate coated with free peptide. Rabbit polyclonal antibody was raised against the synthetic peptide corresponding to the N-terminal sequence of the 8 kDa fragment.

. Figure 8: Immunoprecipitation of the solubilized photoaffinity-labeled SMBP. Solubilized-rat colon membranes photoaffinity-labeled with 1.5 nM [^{125}I]-ICYP-diazirine in the presence of 10 μM 5-HT, 10 μM phentolamine, 20 μM propranolol and

1.1 mM ascorbic acid were immunoprecipitated by 1/200 diluted-preimmunized serum (lane 1) or 10 µg of α8-antibody (lane 2).

. Figure 9: Western blotting of the rat colon membrane proteins. Lane 1 shows control (1/200 diluted-preimmunized serum was used). The 70 kDa band was detected by 2 µg/ml α8-antibody (lane 2). The detection was inhibited when antibody was preincubated with 10 µg/ml specific peptide (lane 3).

. Figure 10: Relationship between the efficacy of β-AR agonists in the rat colon and white adipocytes, in the presence of 10 µM phentolamine and 1 µM propranolol. The linear regression line of the four agonists, except SM-11044, is shown in figure ($r=0.97$, $p < 0.05$). The correlation coefficient, when calculated with SM-11044, was not significant ($r=0.87$, $p > 0.05$). Data represent mean pD2 values ± SEM (from Table 1).

. Figure 11: Time-course of association (○, solid line) and dissociation (●, dashed line) of 1 nM [^{125}I]-ICYP specific binding to rat colon membranes, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Reversibility of binding was obtained by the addition of 100 µM SM-11044 at equilibrium (30 min). Data represent mean of two experiments performed in duplicate.

. Figure 12: Total, non-specific and specific binding of [^{125}I]-ICYP to rat colon membranes, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Non-specific binding was determined in the presence of 100 µM SM-11044. Data represent mean of two experiments performed in duplicate. The inset shows Scatchard's plot of the specific binding ($r=-0.978$, $p < 0.001$). The Kd was 11.0 ± 0.95 nM and the Bmax was 716.7 ± 21.12 fmol/mg protein.

. Figure 13: Displacement of 1 nM [^{125}I]-ICYP specific binding to rat colon membranes by (a) catecholamines, 5-HT and (b) stereo-isomers of SM-11044, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Data represent mean of two to four experiments performed in duplicate.

. Figure 14: SDS-PAGE followed by autoradiography of solubilized rat colon membranes photoaffinity-labeled with 1.5 nM [^{125}I]-ICYP-diazirine in the presence

of 10 µM 5-HT, 10 µM phentolamine and different competitors. Lane 1, control; lane 2, displacement by 20 µM propranolol; lane 3, displacement by 20 µM propranolol and 100 µM BRL-37344; lane 4, displacement by 20 µM propranolol and 100 µM SM-11044.

. Figure 15: Two-dimensional SDS-PAGE followed by autoradiography of solubilized rat colon membranes photoaffinity-labeled with 1.5nM [^{125}I] ICYP-diazirine in the presence of 10 µM 5-HT, 10 µM phentolamine and 20µM propranolol.

. Figure 16: Tryptic cleavage of the photoaffinity-labeled rat colon membranes. The partially purified labeled proteins were incubated without (lane 1) or with 50 µg trypsin (lane 2) for 24 h at 37°C, separated by Tricine-SDS-PAGE and subjected to autoradiography.

. Figure 17: Displacement of 1 nM [^{125}I]-ICYP specific binding to rat skeletal muscle membranes by SM-11044 and (-)isoproterenol, in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol. Data represent mean ± S.E.M of two experiments performed in duplicate.

. Figure 18: Human multiple tissue northern blot hybridized with labeled 300 bp probe. Washes at 2 x SSC, 0.05% S.D.S, at room temperature and exposure on Hyperfilm MP with two intensifying screens at -80°C for three days. (A) Northern blot hybridization was performed on polyadenylated mRNA from 8 different smooth and striated human muscles. (B) similar analysis with a variety of non muscular tissues (heart, brain, placenta, lung, liver, kidney and pancreas). On the left: scale indicates RNA molecular weight marker in kilobases (Kb).

. Figures 19, 20 and 21 illustrate the restriction map of SEQ ID NO:14 (all sites: figure 19; unique sites only: figure 20 and figure 21).

. Figure 22 illustrates a sequence comparison with known proteins (Arabidobsis protein, hMP70 protein, p76 protein, D87444 protein and Emp70 protein).

. Figure 23 illustrates (A) a comparison of hydropathy profiles (Kyte & Doolittle) by GeneJockey Sequence Processor programm between SMBP and the homologous proteins D87444, Hu p76, hMP70 and Emp70 from yeast and Arabidobsis protein. (B) Comparison of the hydropathy profiles (Kyte & Doolittle method) of the C-

terminal hydrophobic region between SMBP and the homologous proteins D87444, Hu p76 and Emp70 of yeast.

Figure 24 illustrates the sequences corresponding to the hydrophobic stretches (boxes).

Figure 25 illustrates immunoprecipitation of (^{125}I)-iodinated cell membrane proteins by α8-antibody: lane 1: COS cells transfected with a vector containing the angiotensin receptor AT2R; lane 2: COS cells transfected with a vector containing the SMBP nucleotide sequence.

Example 1: Isolation and characterization of the instant receptor in rat colon smooth muscle membranes.

1) **Materials and Methods**

SM-11044 ((L)-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl) propyl] serine pyrrolidine amide hydrobromide) and (±)-cyanopindolol were synthesized at Sumitomo Pharmaceuticals (Osaka, Japan). (-)-3-[^{125}I] iodocyanopindolol ([^{125}I]-ICYP) and (±)-3 [^{125}I]-iodocyanopindolol-diazirine ([^{125}I]-ICYP-diazirine) were purchased from Amersham (Buckinghamshire, England). All other materials were reagent grade.

Rat colon membrane preparation

Frozen rat colons (SD strains, male and female) were purchased from Pel-Freeze Biologicals (Arkansas, USA). Membranes from colon smooth muscles were prepared as essentially described by Ek et al., 1986, with the slight following modifications. The colon segment was washed in ice-cold Tris-saline (10 mM Tris/HCl, 154 mM NaCl, (pH 7.4)), cut open longitudinally and the mucosa was removed by scrubbing with a glass slide on ice-cold plastic plate. The smooth muscle preparations were homogenized with a Polytron homogenizer for 1 min. The homogenate was filtered through a gauze and centrifuged (1,500 x g for 20 min at 4°C), the supernatant was collected and centrifuged (50,000 x g for 20 min at 4°C). The pellet comprising the membranes was resuspended in Tris-saline and was stored at -80°C until use. The protein concentration was determined by Bio-Rad protein assay kit (Bio-Rad USA).

Binding assays in rat colon membranes

Saturation binding studies were performed in a final volume of 200 µl of Tris-saline containing 50 µg membrane proteins and different concentrations (0.05-25 nM) of [^{125}I]-ICYP, in the presence of 10 µM serotonine (5-HT), 10 µM phentolamine, 20 µM (±) propranolol and 1.1 mM ascorbic acid (pH 7.4), to block possible 5-HT receptors, ARs and oxidation of catecholamines, respectively. The [^{125}I]-ICYP was used after removing methyl alcohol by compressed air to avoid the influence of the solvent. Incubations were carried out at 37°C for 30 min in a shaking water-bath incubator and terminated by addition of 4 ml of ice-cold Tris-saline followed by rapid filtration under vacuum on Whatman GF/B filter presoaked in Tris-saline containing 0.1% polyethyleneimine (pH 7.4). The filters were washed three times with 4 ml of ice-cold Tris-saline, transferred to plastic tubes and counted in a γ-counter.

Photoaffinity labeling of the rat colon membranes

Photoaffinity labeling was performed in a final volume of 10 ml of Tris-saline containing 50 mg membranes, 0.5 nM [^{125}I]-ICYP-diazirine, supplemented with 10 µM 5-HT, 10 µM phentolamine, 20 µM (±) propranolol and 1.1 mM ascorbic acid (pH 7.4) were incubated at 37°C for 60 min in the dark in a shaking water-bath incubator; the reaction was terminated by addition of 20 ml of ice-cold Tris-saline followed by a rapid centrifugation (50,000 x g for 10 min at 4°C). The membranes were resuspended in 2-3 ml of the same buffer and irradiated with a UV lamp for 10 min with cooling by circulating water (Guillaume et al., 1994). The labeled membranes were diluted with 20 ml of ice-cold Tris-saline, centrifuged (50000 x g for 30 min at 4°C). The labeled membranes were immediately denatured in SDS-reducing buffer (5% SDS, 1% 2β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 50 mM Tris/HCl, pH 6.8) for 1 h or more at room temperature before electrophoresis.

Preparative SDS-PAGE and extraction of the photoaffinity-labelled proteins

Preparative SDS-PAGE was performed with a large size (160 mm width x 200 mm height x 3 mm thickness) of 12% separating and 4% stacking polyacrylamide

gels (40% T, 2.6% C) under reducing conditions essentially according to the methods of Laemmli, 1970. After electrophoresis, the gels were packed in a plastic bag and autoradiographed for 3 days at 4°C on X-OMAT™ AR film (Eastman Kodak Co., USA). The photoaffinity labeled proteins were extracted by passive extraction, as follows. The radioactive 34 kDa band was cut out and crushed to small pieces of less than 3x3x3 mm^3 by squeezing out using 10 ml disposable plastic syringe (Terumo, Japan). The gels were immersed in twice volume of 100 mM Tris/HCl (pH 8.0) containing 0.1% SDS (extraction buffer), and incubated for 16 h at 37°C with rotating. The extract was recovered using a SPIN-XII (0.45 µm pore size, Costar, USA) at 1,500 x g for 30 min. The remaining gel pieces were again immersed in twice volume of extraction buffer, incubated for 2 h at 37°C with rotating, and the extract was recovered as described above. The two extracts were combined and concentrated to at maximum 0.5 ml using Centriprep 10 and Centricon 10 (Amicon, USA) and kept at -20°C.

Chemical cleavage of the extracts from preparative SDS-PAGE and purification by HPLC

The 34 kDa photoaffinity-labeled protein extracted from the preparative SDS-PAGE were washed twice by distilled water using Centricon 10 and lyophilized by vacuum concentrator and treated with 200 µl of 70% formic acid or 10% CNBr/70% formic acid for 24 h at room temperature, or 70% formic acid for 72 h at 37°C in the dark. The cleaved products were diluted with 500 µl distilled water and lyophilized. This washing procedure was repeated three times. The cleaved products were dissolved in SDS-reducing buffer and neutralized by addition of aliquots of 30% NaOH until changing the coloration to blue, and were separated by tricine-SDS-PAGE. The gels were dried and autoradiographed. The labeled bands were cut out, passively extracted and blotted on PVDF membranes by centrifugation (ProSpin™, Applied Biosystems, USA). The membranes were washed 3 times with 1 ml of 20% methanol to remove SDS and salts. The fragments were extracted by 200 µl of 75% hexafluoro-isopropanol. Each elution was dried to 20 µl in vacuum concentrator, dissolved in 75µl DMSO and 75 µl of starting buffer (15% acetonitrile-15% isopropanol-0.5% TFA; buffer A) and loaded on a C4

reverse phase column (Aquapore Butyl BU-300, 2.1 mm ID, 10 mm length, Applied Biosystems). Separation was carried out by a 120 min gradient elution at 40°C with 50% acetonitrile-50% isopropanol containing 0.5% TFA (buffer B) at a flow rate of 0.35ml/min using a Waters 625 LC System. The gradient started from 30% to 98% buffer B. The elution of fragments was monitored by the absorbance at 210 and 275nm, and the elution of radioiodinated products was monitored by γ-counting of the fractions.

Tricine-SDS-PAGE

Chemical cleaved fragments were separated on a Tricine gel system under reducing conditions (Schägger H. et al., 1987) using 18% polyacrylamide separating gel containing 10.7% glycerol. The gels aged for 16 h to allow for decomposition of reactive chemical intermediates after polymerization.

Amino acid sequencing

Amino acid sequence determination was performed by Edman degradation, 1967, with an Applied Biosystems 473A protein sequencer. Samples were applied to precycled filters, coated with Polybrene (Biobrene, Applied Biosystems) to reduce peptide-wash-out and to improve initial yields.

Antibody preparation

Antibody was prepared as essentially described by Guillaume et al. (Eur. J. Biochem., 1994, **224**, 761-770).

Briefly, based on the determined amino acid sequences, peptides were synthesized adding a cysteine residue at C-terminal residue to facilitate coupling to the carrier protein (Keyhole limpet hemocyanin, KLH). The synthetic peptides were conjugated to KLH through their cysteine residues. A 0.4 mg of the peptide-conjugate, suspended in Freund's complete adjuvant, was intradermally injected into rabbit. Boosters were given 4 times at 2 weeks intervals by injection of a 0.2 mg of the peptide-conjugate suspended in Freund's incomplete adjuvant. Two weeks later the final immunization, antiserum was recovered from whole blood.

Antibody was purified by affinity chromatography on a column containing the synthetic peptide coupled to activated thiol-Sepharose-4B (Pharmacia)

through a cysteine at C-terminal residue, and the antibody titer level against the free peptide without conjugation to KLH was determined by ELISA.

Immunoprecipitation

Total amounts of 10 mg membranes were photoaffinity-labeled with 1.5 nM [^{125}I]-ICYP-diazirine in the presence of 10 µM, 5-HT, 10 µM phentolamine, 20 µM propranolol and 1.1 mM ascorbic acid in 10 ml of Tris-saline (pH 7.4). Membranes were solubilized at 1 mg membrane protein/ml of Tris-saline containing 2% n-octylglucoside (n-octyl β-D-glucopyranoside, Sigma) for 2 h on ice with occasional mixing. The solubilized-proteins were separated from the insoluble material by centrifugation (200,000 x g, 30 min at 4°C). The proteins were treated with 8 M urea for 1 h at room temperature with occasional mixing and were washed 5 times with Tris-saline using Centricon 10. The solubilized-membrane proteins were dissolved in 1ml Tris-saline containing 0.1% Tween-20 and were incubated with 10 µg antibody and 50 µl protein-A-agarose beads (Boehringer-Mannheim, Germany) for 16 h at 4°C with rotating. The precipitant was gently washed 5 times with ice-cold Tris-saline containing 0.1% Tween-20 and denatured in SDS-reducing buffer for more than 1 h at room temperature. The immunoprecipitated proteins were subjected to 12% SDS-PAGE and autoradiographed.

Western blotting

Photoaffinity-labeled membranes (40 µg protein) were separated by 12% SDS-PAGE. Electrotransfer of proteins onto nitrocellulose was carried out essentially according to Towbin et al., 1979, on a Trans-Blot SD apparatus (Bio-Rad) for 1 h at a current intensity of 1mA/cm^2. Nitrocellulose membranes were washed three times with Dulbecco's phosphate buffered saline (PBS) containing 0.2% Tween-20 and were saturated in PBS containing 5% skimmed milk powder and 0.2% Tween-20 for 1 h at room temperature. Antibody (2 µg/ml in PBS containing 1% skimmed milk powder and 0.2% Tween-20; buffer C) was allowed to react for 16 h at 4°C.

After three times washing in buffer C, the nitrocellulose strips were incubated for 45 min at room temperature with peroxidase-conjugated affinity-purified Goat anti-rabbit IgG (Jackson Immuno-Research Laboratories, USA) at a 1/2500 dilution in

buffer C, washed three times in buffer C. After washing in PBS containing 0.2% Tween-20, reactive bands were visualized with an ECL kit (Amersham, England). In inhibition experiments, antibody was preincubated for 2 h at 37°C with free peptide at a concentration of 10 µg/ml in buffer C.

2) Results

Extraction of the photoaffinity-labeled SMBP

Membrane proteins of 2.0 g were collected from 600 rat colon smooth muscles. The ligand binding activity of SMBP was assessed by [^{125}I]-ICYP under blockade of adrenergic and serotonine receptors. Scatchard plot analysis revealed a single class of binding sites with a dissociation constant (Kd) of 7.22 ± 0.007nM and a maximum number of binding sites (Bmax) of 1.13 ± 0.071 pmol/mg membrane protein (two independent experiments performed duplicate, expressed as means ± SD).

The SMBP was too hydrophobic to separate by any column chromatography such as reverse-phase HPLC with C4 column (Aquapore Butyl BU-300, Applied Biosystems), ion exchange chromatography (Aquapore Weak Anion AX-300, Applied Biosystems), chromatofocusing (PBE 94 and Polybuffer 74, Pharmacia), hydroxyapatite chromatography (BioGel HPHT, Bio-Rad). Preparative SDS-PAGE was performed to separate SMBP just after the photoaffinity labeling. Fifty mg of the labeled-membranes could be loaded on a set of polyacrylamide gels without serious diffusion of the 34 kDa labeled-SMBP (figure 1). The passive extraction of 34kDa bands yielded 79.3-86.2% of the total radioactive proteins in gels.

Chemical cleavage, purification and sequencing

Chemical cleavage has some advantage in contrast to proteolytic digestion; it avoids contamination by protease itself, and produces limited numbers of large fragments. Analytically, each 1 mg of the labeled 34 kDa protein was treated with 10% CNBr in 70% formic acid or in 75% TFA to compare the effect of acid. In formic acid condition, CNBr generated three labeled fragments of 8, 10 and 12 kDa, and formic acid alone generated a single 8 kDa labeled fragment. In the acid condition with TFA, most of

the labeling was dissociated by acid itself, a single 10 kDa labeled fragment was observed by CNBr cleavage (figure 2).

The extract of the labeled 34 kDa protein from 400 mg membranes (411794 cpm) was preparatively cleaved by CNBr/formic acid, and an aliquot of the cleaved-products was resolved on tricine-SDS-PAGE gels. Three labeled fragments of major 12 kDa and minor 8 and 10 kDa were observed on autoradiogram of coomasie blue stained gels (figure 3).

Cleavage at methionine residues by CNBr/formic acid treatment for 24 h at room temperature of the photoaffinity-labeled 34 kDa protein yielded three labelled-fragments (8, 10 and 12 kDa, Fig. 4a, lane 3). Treatment by formic acid alone generated a single 8 kDa fragment (Fig. 4a, lane 2), and the density of the 8 kDa band increased upon prolonged incubation (for 72 h at 37°C, Fig. 4b, lane 2).

The extract of the labeled 34 kDa protein from 400 mg membrane (381198 cpm) was preparatively cleaved by formic acid, and an aliquot of the cleaved-products was resolved on tricine SDS-PAGE gels. A single labeled-fragment of 8kDa was observed on autoradiogram of coomasie blue stained-gels (figure 5). The radioactive 8 kDa fragment (total 21400 cpm) in preparative scale was extracted by passive extraction from tricine-SDS-PAGE gels without coomasie blue staining, and was blotted on PVDF membranes (19581 cpm). The fragment was extracted from PVDF membranes (10045 cpm) and further purified by reverse-phase HPLC. One radioactive peak was observed at 62% buffer B (fraction n° 27 and 28; total 3239 cpm, figure 6). Total recovery yield of the initial radioactivity was 91.6%. The peak fractions were submitted to protein sequencer, and the resulting amino acid sequence was determined as follows:

```
1     5     10    15
|     |     |     |
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(D) P X F F Q H R I H V F S I F N H (SEQ ID NO:6)

Parenthesis; expected amino acid

X; undetermined amino acid.

Analytical CNBr-cleavage indicated that cleavage at methionine residue in the presence of TFA, which improve the cleave at CNBr-resistant bonds such as Met-Thr or Met-Ser (Fontana A. et al., 1986), generated a single 10 kDa fragment.

In formic acid condition, CNBr generated three labeled fragments of 8, 10 and 12 kDa, and formic acid alone generated a single 8 kDa labeled fragment. These data suggest that 12 kDa fragment contains a CNBr-resistant methionine residue cleaved to 10 kDa by CNBr/TFA and that the 8 kDa fragment by formic acid alone is a product by cleavage at acid-sensitive bond such as Asp-Pro.

Immunoprecipitation and Western blotting

The peptide corresponding to the N-terminal sequence of the 8 kDa fragment (Acetyl-FFQHRIHVFSIFNHC) was coupled to KLH and the conjugate was used to raise antibody with high titer. The antibody response was observed at $2x10^{-5}$ dilution of antiserum and at 0.08 µg/ml of affinity purified antibody (α8-antibody) as assessed by ELISA against free peptide without conjugation to KLH (figure 7).

The synthetic peptide corresponding to the 8 kDa-fragment was hydrophobic and could not be dissolved in a buffer without dimethyl sulfoxide. Initially, the labeled 34 kDa protein, extracted from preparative SDS-PAGE gels, was used after removing SDS, but no labeled protein was immunoprecipitated. After solubilization of the photoaffinity-labeled membranes by n-octylglucoside followed by denaturation with urea, the α8-antibody immunoprecipitated the labeled 34 kDa SMBP (figure 8).

The α8-antibody recognized only a 70 kDa band by western blotting. The specificity of the antibody was demonstrated by the ability of the free peptide to inhibit the binding of the antibody to the 70 kDa protein (figure 9). In a separate experiment, photoaffinity-labeled SMBP was purified by two-dimensional electrophoresis in preparative scale, and the 34 kDa labeled spot in gels was isolated, extracted and subjected to SDS-PAGE. Two labeled bands of 34 and 70 kDa derived from 34 kDa were observed, indicating that the 70 kDa protein could be dimer.

Example 2: Pharmacological properties of the rat receptor according to example 1.

Catecholamine-induced relaxant responses which are resistant to blockade of α-, β1- and β2-adrenoceptors (ARs) have been described in a number of gastro-intestinal smooth muscle preparations, such as guinea pig ileum (Bond R.A. et al., 1988), rat proximal colon (Croci T. et al., 1988), rat distal colon (McLaughlin D.P. et al., 1990), rat gastric fundus (McLaughlin D.P. et al., 1991) and rat jejunum (Van der Vliet A. et al., 1990). Manara et al., 1990, actually reported that the phenylethanolamino-tetralines-stimulated rat colon relaxation paralleled rat adipocyte lipolysis, suggesting that this response predominantly involved the β3-AR.

1) **Materials and Methods**

Chemicals

SM-11044 ((L)-threo-3-(3,4-dihydroxyphenyl)-N-[3-(4-fluorophenyl) propyl] serine pyrrolidine amide hydrobromide), SM-14786 ((D)-threo isomer of SM-11044), SM-14011 ((DL)-threo-isomer of SM-11044), SM-14010 ((DL)-erythro-isomer of SM-11044), BRL-35135A ((R*R*)-(+)-4-[2'-[2-hydroxy 2-(3-chlorophenyl) ethyl amino] propyl] phenoxyacetic acid methyl ester), BRL-37344 (acid metabolite of BRL-35135A) and ((125I)-cyanopindolol were synthesised at Sumitomo Pharmaceuticals (Osaka, Japan). CGP-12177A and CGP-20712A were gifts from Ciba-Geigy Corporation (Basal, Switzerland). ICI-198157 ((RS)-4-[2-[(2-hydroxy-3-phenoxypropyl) amino] ethoxy] phenoxyacetic acid methyl ester), ICI-201651 (acid metabolite of ICI-198157) and ICI-215001 ((S)-isomer of ICI-201651) and ICI-118551 were obtained from Zeneca Pharmaceuticals (Macclesfield, England). SR-58611A ((RS)-N-(7 carbethoxymethoxyl-1,2,3,4-tetrahydronaphth-2-yl)-2-hydroxy-2-(3 chlorophenyl) ethanamine hydro-chloride) was a gift from Sanofi-Midy (Milano, Italy). (+)-Carazolol was obtained from Boehringer Mannheim (Mannheim, Germany). (+)-Bupranolol was a gift from Schwarz Pharma (Monheim, Germany). (-)-3-[125I] iodocyanopindolol ([125I]-ICYP) and (+)-3 [125I]-iodocyanopindolol-diazirine ([125I]-ICYP-diazirine) were purchased from Amersham (Buckinghamshire, England). All other drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Relaxation of rat colon

Rat colon segment (2 cm) was suspended in organ bath containing 10 ml modified-Tyrode solution (Sugasawa T. et al., Eur. J. Pharmacol., 1992, 216, 207-215). The Tyrode solution contained 0.5 μM atropine, 0.5 μM desmethylimipramine, 30 μM hydrocortisone, 30 μM ascorbic acid, 10 μM phentolamine and 1 μM propranolol throughout study, in order to inhibit spontaneous contraction, neuronal and extra-neuronal uptake of norepinephrine, oxidation of catecholamines, possible α, β1- and β2-AR effects, respectively.

The relaxant action of agonists was determined by measuring relaxation of KCl (100 mM)-induced tonus evoked by cumulative addition of the agonists as described previously (Sugasawa T. et al. cited above). In the case of testing the effect of cyanopindolol, it was added 5 - 10 min before the addition of agonist.

Lipolysis in rat white adipocytes

White adipocytes were isolated from epididymal fat pads of male Wistar rats (190 - 230 g) and lipolysis was determined according to the previous report (Sugasawa T. et al. cited above). The cells were preincubated for 5 min at 37°C in the presence of 30 μM ascorbic acid, 10 μM phentolamine and 1 μM propranolol.

Agonists were then applied and incubated for 90 min. In the case of testing the effect of cyanopindolol, it was added 5 min before the addition of agonist.

Schild plot

Agonist concentration-ratios (CR) were determined from the EC_{50} values of the concentration-response curves of agonists with or without cyanopindolol, according to the method of Arunlakshana et al., 1959.

Linear regression analysis was used to estimate the pA_2 value and slope of the line, after confirming that the regression was linear and the slope was not significantly different from unity (Cochran-cox test, $p > 0.05$). The EC_{50} values were calculated using the computer program, InPlot™.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance between two data sets was examined by Student's t-test or Cochran-cox test, depending on the homogeneity of the variances. Duncan's multiple range test was used for multiple data sets. A probability level of $p < 0.05$ was considered to be significant.

Membrane preparation

Membranes from the colon smooth muscle and from skeletal muscle were prepared from male Wistar rats (300 - 360 g) as essentially mentioned in example 1.

Binding assays in membranes

Saturation binding studies were performed in a final volume of 200 µl of Tris-saline containing 50 µg membrane proteins and different concentrations (0.05-25 nM) of [^{125}I]-ICYP, supplemented with 10 µM serotonine (5-HT), 10 µM phentolamine, 20 µM propranolol and 1.1 mM ascorbic acid (pH 7.4), to block possible 5-HT receptors, ARs and oxidation of catecholamines, respectively. The [^{125}I]-ICYP was used after removing methyl alcohol by compressed air to avoid the influence of the solvent. Incubations were carried out at 37°C for 30 min in a shaking water-bath incubator and terminated by addition of 4 ml of ice-cold Tris-saline followed by rapid filtration under vacuum on Whatman GF/B filter presoaked in Tris-saline containing 0.1% polyethyleneimine (pH 7.4). The filters were washed three times with 4 ml of ice-cold Tris-saline, transferred to plastic tubes and counted in a γ-counter.

Competition assays were performed against 1 nM [^{125}I]-ICYP. Non-specific binding was determined in the presence of 100 µM SM-11044. The inhibition constant, Ki, of a ligand was calculated using the equation described by Cheng and Prusoff (Biochem. Pharmacol., 1973, **22**, 3099-3108). Hill coefficient was calculated by linear regression using saturation experiment data. Pseudo-Hill coefficient and IC_{50} were determined by the computer program, InPlot™ (GraphPad Software, CA, USA).

Photoaffinity labeling of the membranes

Photoaffinity labeling was performed in a final volume of 1 ml of Tris-saline containing 0.5 mg membranes, 1.5 nM [^{125}I]-ICYP-diazirine, supplemented with

10 µM 5-HT, 10 µM phentolamine, 20 µM propranolol and 1.1 mM ascorbic acid (pH 7.4). Incubations were carried out in the presence or absence of competitor at 37°C for 45 min in the dark in a shaking water-bath incubator and terminated by addition of 10 ml of ice-cold Tris-saline followed by a rapid centrifugation (150,000 x g for 10 min at 4°C). The membranes were irradiated with a UV lamp for 5 min with cooling by circulating water. The labeled membranes were diluted with 10 ml of ice-cold Tris-saline, centrifuged (150,000 x g for 30 min at 4°C), and the pellet was resuspended in Tris-saline and kept at -80°C.

SDS-PAGE

SDS-PAGE was performed under reducing conditions essentially as described by Laemmli, 1970, using 12% polyacrylamide gels (40% T, 2.6% C). The photoaffinity-labelled membranes were incubated in SDS-sample buffer (5% SDS, 1% 2β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 50 mM Tris/HCl, (pH 6.8)) for at least 1 h at room temperature. After electrophoresis, the gels were dried and autoradiographed on X-OMAT™ AR film (Eastman Kodak Co., NY, USA), as specified in example 1, chapter «preparative SDS-PAGE».

Two-dimensional PAGE of photoaffinity-labeled membranes

Photoaffinity-labeled membranes in the presence of 10 µM 5-HT, 10µM phentolamine and 20 µM propranolol were solubilized in IEF-sample buffer (8 M urea, 0.3% SDS, 5.6% Triton X-100, 2.8% 2β-mercaptethanol, 1.1% Bio-Lyte 5/8 ampholyte and 0.6% Bio-Lyte 8/10 ampholyte (Bio-Rad)) and 30 µg of membrane proteins were submitted to IEF electrophoresis in a 5 - 10 pI range of 4% polyacrylamide tube gels containing 2.0% Bio-Lyte 5/8 ampholyte, 1.0% Bio-Lyte 8/10 ampholyte, 8 M urea and 2% Triton X-100. The second dimension was conducted on SDS-PAGE of 9% polyacrylamide gels. The gels were then dried and submitted to autoradiography as described above.

Cleavage by endoglycosidase or N-glycopeptidase F

Photoaffinity-labeled membranes in the presence of 10 µM 5-HT, 10µM phentolamine and 20 µM propranolol were treated with N glycopeptidase F (PNGase F,

EC 3.2.2.18) or endoglycosidase (Endo Hf, EC 3.2.1.96), using kits according to the manufacturer's specifications (New England Bio-Labs, MA, USA). Briefly, the membranes were solubilized in 0.5% SDS and 1% 2β-mercaptethanol, and 40 µg of membrane proteins were incubated with 5000 units of PNGase F in the presence of 1% NP-40 or with 2000 units of Endo Hf for 3 h at 37°C. The digested samples were subjected to SDS-PAGE of 12% polyacrylamide gels. The gels were then dried and submitted to auto-radiography as described above.

Wheat germ agglutinine (WGA) - sepharose chromatography

Photoaffinity-labeled membranes in the presence of 10 µM 5-HT, 10µM phentolamine and 20 µM propranolol were solubilized in 1% Triton X 100 in Tris-saline at 4°C for 16 h. The solubilized material was collected after centrifugation (200,000 x g for 1 h at 4°C) and diluted to 0.1% Triton X-100 by Tris-saline. One milliliter gel bed volume of WGA-sepharose 6MB (Sigma) was washed and equilibrated with 30 ml of 0.1% Triton X-100 in Tris-saline (buffer A), and 1 ml of solubilized material containing 200 µg membrane proteins was loaded at room temperature. The unretained fraction was recycled three times. After washing with 10 ml of buffer A, the bound material was eluted with 5 ml of 300 mM N-acetyl-D-glucosamine (Merck) in buffer A. The fractions were subjected to SDS-PAGE of 12% polyacrylamide gels. The gels were then dried and submitted to autoradiography as described above.

Tryptic cleavage

The photoaffinity-labeled membranes were subjected to SDS-PAGE of 12% polyacrylamide gels. The gels were then dried and submitted to autoradiography as described above. The radioactive band at 34 kDa was excised, immersed in distilled water and minced to small pieces (2 mm width x 2 mm height). The isolated gel pieces corresponding to 800 µg membrane proteins was digested in 500 µl of 100 mM Tris/HCl (pH 8.0) containing 0.1% SDS and 50 µg trypsin (EC 3.4.21.4, Type IX from Porcine Pancreas, Sigma) for 24 h at 37°C according to the method of Kawasaki H. et al., 1990. After digestion, the supernatant was recovered and filtrated using a SPIN-X filter (0.45 mm pore size, Costar, MA, USA). The gel pieces were crushed through a nylon mesh (200

mesh) by centrifugation for 10 min at 14,000 x g. A 2-fold volume of 100 mM Tris/HCl containing 0.1% SDS was added to the crushed gels, and a second extraction was performed by incubation for 2 h at 37°C with rotating. After incubation, the supernatants were recovered by SPIN-X filter. The two extracts were combined, vacuum concentrated and submitted to Tricine-SDS-PAGE.

Chemical cleavage

The 34 kDa photoaffinity-labeled protein was isolated by SDS-PAGE and extracted with 100 mM Tris/HCl (pH 8.0) containing 0.1% SDS as described above. The extracts were combined and concentrated by Centricon 10 (Amicon, MA, USA) and washed twice by distilled water. The extracts were lyophilised by vacuum concentrator and treated with 200 µl of 70% formic acid or 1% CNBr/70% formic acid for 24 h at room temperature, or 70% formic acid for 72 h at 37°C in the dark. The cleaved products were diluted with 500 µl distilled water and lyophilised. This washing procedure was repeated three times. The cleaved products were separated by Tricine SDS-PAGE.

Tricine-SDS-PAGE

Tryptic and chemical cleaved fragments were separated on a Tricine gel system under reducing conditions (Schägger H. et al., 1987) using 18% polyacrylamide separating gel containing 10.7% glycerol. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250 (Sigma) in 40% methanol and 10% acetic acid, and destained in 10% acetic acid. The gels were then dried and submitted to autoradiography as described above.

2) **Results**

Functional studies in rat colon and white adipocytes

Under blockade of α-, β1- and β2-ARs (in the presence of 10 µM phentolamine and 1 µM propranolol), a number of β–AR agonists relaxed KCl-induced tonus in rat colon smooth muscle segment, giving a rank order of potency of BRL-37344 > SM-11044 >> isoproterenol >> norepinephrine = epinephrine (Table 1).

TABLE 1

Agonist efficiency in rat colon relaxation and rat white adipocyte lipolysis in the presence of 10 μM phentolamine and 1 μM propranolol

Agonist	Rat colon			Rat white adipocytes		
	pD_2	IA	n	pD_2	IA	n
(-)-isoproterenol	6.64±0.22	1.00±0.063	5	5.86±0.07	1.00±0.037	5
(-)-norepinephrine	5.85±0.27	0.85±0.168	4	5.40±0.10	1.02±0.058	5
(-)-epinephrine	5.92±0.06	0.86±0.137	6	5.16±0.06	0.91±0.036	5
BRL-37344	7.50±0.18	1.00±0.126	8	7.25±0.09	0.72±0.0333**	5
SM-11044	7.29±0.21	1.48±0.166*	7	5.96±0.11	0.86±0.054	5

Statistical significance between IA values; * $p < 0.05$, ** $p < 0.01$ vs isoproterenol (Duncan's multiple range test).

The IA value of SM-11044 was significantly higher than that of isoproterenol (Duncan's multiple range test, $p < 0.05$), indicating different modes of action. In rat white adipocytes, the same agonists stimulated lipolysis with a rank order of potency of BRL-37344 >> SM-11044 = isoproterenol > norepinephrine > epinephrine (Table 1). The linear regression line for isoproterenol, norepinephrine, epinephrine and BRL-37344 reveals a significant correlation ($r=0.97$, $p < 0.05$) between agonist induced rat colon relaxation and adipocyte lipolysis (Fig. 10), suggesting that both effects predominantly involve the same atypical β-, that is β3-AR stimulation. In contrast to the four ligands, SM-11044 stimulated colon relaxation more efficiently than adipocyte lipolysis (Fig. 10). Indeed, the correlation coefficient ceased to be significant when linear regression was analyzed with all agonists including SM-11044 ($r= 0.87$, $p > 0.05$). These data suggest that SM-11044 acts on β3-AR and additional functional site that mediates relaxation in rat colon. Antagonism of cyanopindolol for SM-11044 and for isoproterenol was compared in both preparations. Cyanopindolol itself, up to the concentration of 10 μM used here, had no effect on the degree of tonus induced by KCl in rat colon and did not stimulate lipolysis in rat white adipocytes. Cyanopindolol antagonised agonist-induced rat colon relaxation in a concentration-dependent manner, with pA_2 values for SM-11044 of 8.31 (slope = 0.78) and for isoproterenol of 7.65 (slope = 1.03) (Table 2).

TABLE 2

pA_2 values for cyanopindolol in rat colon and rat white adipocytes in the presence of 10 μM phentolamine and 1 μM propranolol.

Agonist	Rat colon			Rat white adipocytes		
	pA_2	Slope	n	pA_2	Slope	n
(-)-isoproterenol	7.65±0.48	1.03±0.08	5	7.44±0.61	1.08±0.10	4
SM-11044	8.31±0.88	0.78±0.11	5	7.32±1.51	0.96±0.21	4

Cyanopindolol also antagonized agonist-induced rat white adipocyte lipolysis in a concentration-dependent manner, with pA_2 values for SM 11044 of 7.32 (slope = 0.96) and for isoproterenol of 7.44 (slope = 1.08) (Table 2). The similar pA_2 values for isoproterenol in colon (7.65), SM-11044 in adipocytes (7.32) and isoproterenol in adipocytes (7.44) with the slopes close to unity, indicating the competitive antagonism of cyanopindolol for both agonists binding to β3-AR. All slopes of Schild plots were not significantly different from unity. However, only the slope for SM-11044 in rat colon (0.78) seemed to be lower than unity with high pA2 value (8.31), suggesting that SM-11044 and cyanopindolol compete not only binding to β3-AR but also to additional functional site on rat colon.

Binding assays in rat colon membranes

In order to identify the predicted functional site, being competed by SM-11044 and cyanopindolol, binding studies in rat colon smooth muscle membranes were performed using [^{125}I]-ICYP for radioligand and SM-11044 for non-specific binding determination, under blockade of serotonine, α-, β1-, β2- and also β3-adrenergic receptors (in the presence of 10 μM 5-HT, 10 μM phentolamine and 20 μM propranolol). The time course of specific binding of [^{125}I]-ICYP (1 nM) to rat colon membranes was illustrated in Fig. 11. Specific binding achieved equilibrium levels at 30 min (82.7 ± 1.9%, n=2), and was reversed by addition of SM-11044. The results of a saturation experiment with increasing amount of [^{125}I]-ICYP, carried out at equilibrium (30 min incubation), are illustrated in Fig. 12. Scatchard plot analysis revealed a single class of binding sites with a

dissociation constant (Kd) of 11.0 ± 0.95 nM, and a maximum number of binding sites (Bmax) of 716.7 ± 21.12 fmol/mg protein ($r=-0.978$, $p < 0.001$). Hill plot analysis of the saturation curve yielded a coefficient of 0.99 ± 0.03 ($r=0.998$, $p < 0.0001$), indicating the absence of cooperativity.

In competition binding studies, specific binding was not displaced by isoproterenol, norepinephrine, epinephrine, dopamine nor 5-HT, up to the concentration of 1 mM (Fig. 13a, Table 1). The competition binding by isomers of SM-11044 was stereo-selective, SM-14011 (the racemic threo isomer, Ki 2.0 µM) being 15 times more effective than SM-14010 (the racemic erythro-isomer, Ki 29.3 µM) (Fig. 13b, Table 3). The β1-AR antagonist, CGP20712A and the β3-AR agonist, BRL-37344 did not displace the specific binding up to the concentration of 100 µM; the β2-AR antagonist, ICI-1 18551 was effective with a relatively high Ki (28.5 µM) (Table 3). Cyanopindolol was the most effective competitor with a Ki of 0.11 µM, and pindolol had no effect up to the concentration of 100 µM. Carazolol, a ligand structurally related to cyanopindolol, was less effective, in spite of being more lipophilic (Table 3). Interestingly, BRL-35135A (methyl ester of BRL-37344) and ICI 198157 (methyl ester of ICI-201651; ICI-215001, a (S)-enantiomer of ICI-201651) displaced the specific binding, whereas the corresponding acid metabolites were inactive (Table 3). The specific binding was significantly reduced by GTP (29.8 ± 2.7% inhibition at 300 µM ($p < 0.01$) and 98.2 ± 1.3% at 1 mM ($p < 0.001$), n=2, respectively).

TABLE 3

Affinity (Ki) values of various ligands on [^{125}I]-ICYP specific binding to rat colon membranes in the presence of 10 µM 5-HT, 10 µM phentolamine and 20 µM propranolol.

Ligands	Ki (µM)	Pseudo-Hill coefficient
Catecholamines and 5-HT		
(-)-isoproterenol	> 1000	
(-)-norepinephrine	> 1000	
(-)-epinephrin111e	> 1000	
Dopamine	> 1000	
5-HT	> 1000	
SM-11044 and stereo-isomers		
SM-11044 ((l)-threo)	1.8±0.3	1.00±0.12
SM-14786 ((d)-threo)	3.7±0.4	0.92±0.15
SM-14011 ((dl)-threo)	2.0±0.5	1.07±0.15
SM-14010 ((dl)-erythro)	29.3±10.3	0.67±0.13
β1-antagonist		
CGP-20712A	> 100	
β2-antagonist		
ICI-118551	28.5±3.6	0.89±0.14
β3-agonists		
BRL-35135A (ester)	1.4±0.1	0.80±.0.14
BRL-37344 (acid metabolite)	> 100	
ICI-198157 (ester)	29.4±8.9	0.96±0.23
ICI-215001 (acid metabolite)	> 100	
ICI-201651 (acid metabolite)	> 100	
SR-58611A (ester)	5.9±1.0	1.21±0.21
β1-, β2-antagonists having β3-partial agonist potencies		
CGP-12177A	> 100	
(±)-cyanopindolol	0.11±0.02	1.01±0.14
(±)-pindolol	> 100	
(±)-carazolol	8.1±1.7	0.77±0.11
(±)-alprenolol	13.3±2.4	0.85±0.24
β1-, β2-, β3-antagonist		
(±)-bupranolol	11.3±0.8	1.08±0.08

Photoaffinity labeling study

Photoaffinity labeling was performed to visualize the specific binding site in rat colon membranes using [^{125}I]-ICYP-diazirine. In the presence of 10 µM 5-HT and 10 µM phentolamine, but in the absence of propranolol, a single dense band of 34 kDa was visualized in addition to two broad bands with apparent molecular masses of 50 and 70 kDa. (Fig. 14, lane 1). In contrast, in the presence of 20 µM propranolol, 10 µM 5-HT and 10 µM phentolamine, that is, in the same conditions of the competition binding assay with [^{125}I] ICYP, only the 34 kDa band remained visible (Fig. 14, lane 2). These results suggest that the two broad bands are β-ARs. Moreover, the 34 kDa band was not displaced by 100 µM BRL-37344, but was displaced by 100 µM SM-11044 (Fig. 14, lanes 3 and 4, respectively). These data support the results of the competition binding assay, suggesting the existence of a single specific binding site for [^{125}I]-ICYP and SM-11044.

Two-dimensional PAGE of the photoaffinity-labeled membranes confirmed the labeling of a single 34 kDa polypeptide chain corresponding to a pI of 6.0 (Fig. 15). The molecular size of the photoaffinity-labeled 34 kDa protein was not modified by the enzymatic treatments with endoglycosidase or N glycopeptidase F, whereas both enzymes reduced the molecular size of ovalbumin from 43 kDa to 40 kDa. Solubilized photoaffinity-labeled 34 kDa protein (373,298 cpm), were applied to a WGA-sepharose column. The unretained fraction contained 35.7% of the radioactivity, and washed out fractions contained 53.3% of the radioactivity. The specific sugar, 300 mM N-acetyl-D-glucosamine, eluted only 2.3% of the radiolabeled material. The eluted fraction was subjected to SDS-PAGE after concentration, but the photoaffinity-labeled 34 kDa band was not detected. A single 7 kDa labeled-peptide was generated upon digestion of the photoaffinity-labeled 34 kDa protein with trypsin (Fig. 16). Recovery yields in final extracts from the gel pieces were 62.7% for the labeled 34 kDa protein and 90.4% for the in-situ generated tryptic peptides.

Binding studies in rat skeletal muscle membrane preparation

[^{125}I]-ICYP specific binding to skeletal muscle membranes was not displaced by isoproterenol up to concentrations of 10^{-4} M. In contrast, SM-11044 displaced the binding in a concentration-dependent manner (Figure 17).

Pharmacological definition of the instant receptor

- SM-11044, a β-AR agonist, showed atypical agonist effects such as relaxant responses in guinea pig ileum and rat colon intestines, and inhibition of guinea pig eosinophil chemotaxis.

Cyanopindolol competitively antagonized the responses to isoproterenol and SM-11044 at β3-AR with similar pA_2 values (7.32 ~ 7.65) in rat colon intestinal segments and rat white adipocytes. The values were also similar to those reported at β3-AR on rat white adipocytes (Kirkham D. et al., 1992), rat colon, rat gastric fundus (McLaughlin and MacDonald, 1989, 1990), and guinea pig ileum (Blue D.R. et al., 1989). In contrast, cyanopindolol antagonized the additional atypical effect of the SM-11044-induced colon relaxation with higher pA_2 value (8.31) along with low slope of Schild plots (0.78). The results demonstrated the existence of at least two different affinity sites including β3-AR in rat colon. Thus, cyanopindolol and SM-11044 competed not only at β3-AR but also at another atypical binding site. SM-11044 stimulated relaxant responses of the KCl-induced depolarized colon tonus through both sites.

Initial comparison with atypical effects between guinea pig ileum and rat white adipocytes could not exclude species-related difference. However, the difference of atypical effects between rat white adipocytes and rat colon intestines are now evident, that is not species-related phenomenon.

- Detection of the binding site: radioligand binding assay was performed using rat colon smooth muscle membranes based on the results in functional studies that SM-11044 and cyanopindolol competed the sites. In general, if same origin of ligands are used for both radioligand and «cold» ligand, physically- or chemically-related non-specific binding can not be excluded. Furthermore, pA_2 value of cyanopindolol was 8.31 and pD_2 value of SM-11044 was 7.29 in rat colon, suggesting 10-fold higher affinity of cyano-

pindolol than that of SM-11044 at the two atypical components (β3-AR and another site). Therefore, [^{125}I]-ICYP and SM-11044 were used as radioligand and «cold» ligand, respectively.

[^{125}I]-ICYP can bind to β1-, β2-, β3-ARs, serotonine 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors (Tate K.M. et al., 1991; Hoyer D. et al., 1994). In contrast, specific binding was obtained under blockade of these known receptors. Competition binding studies revealed that the binding site was indeed different from these receptors. Natural AR ligands (epinephrine and norepinephrine) and classical β-AR ligand (isoproterenol) showed no affinity, suggesting that the binding site is different from ARs. Several synthetic β-AR ligands including β3-AR agonists (BRL-35135A, SR-58611A and ICI-198157) showed affinity. Atypical effects that could not be explained by β3-AR can be resolved by the existence of this binding site. Indeed, similar binding sites under blockade of β-ARs and serotonine receptors were observed in rat skeletal muscle membranes.

- Biochemical characterization by photoaffinity-labeling study

The binding site in rat colon smooth muscle membranes was visualized by [^{125}I]-ICYP-diazirine, a photoaffinity ligand corresponding to [^{125}I]-ICYP. The apparent molecular size of the site was 34 kDa with an isoelectric point (pI) of 6.0. Deduced molecular sizes of rat β-ARs, serotonine 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors are 43.2 ~ 50.5 kDa (β1-AR, 50.5 kDa; β2-AR, 46.9 kDa; β3-AR, 43.2 kDa; 5-HT$_{1A}$, 46.4 kDa, 5-HT$_{1B}$, 43.2 kDa) (Machida et al., 1990; Gocayne et al., 1987; Muzzin P. et al., 1991; Granneman JG. et al., 1991; Albert A. et al., 1990; Fujiwara et al., 1990, Voigt et al., 1991). In cells or tissues, these receptors are normally glycosylated, then the sizes are usually bigger than the deduced sizes. In contrast, the size of 34 kDa seemed to be smaller than these cloned rat receptors. One explanation may be devoid of N-linked glycosylation. The isoelectric point indicates that the binding site is an acidic protein like β-ARs (Fraser C.M., 1984). Chemical cleavage at mostly methionine residues resulted in 10 and 12 kDa, and acid cleavage at mainly asparagine-proline bonds resulted in 8 kDa, indicating this protein contains methionine residues and may include asparagine-proline bonds.

Example 3: Isolation and characterization of the instant receptor in human skeletal muscle.

- Preparation of probes:

SEQ ID NO:6 has been compared to GenBank and EMBL data base by tblastn program (Altschul S.F. et al., 1990); in dbest data base, a human expressed sequence tag (EST) with almost 100% homology with SEQ ID NO:6 was found; it corresponds to SEQ ID NO:5, found in *H. sapiens* as a partial cDNA sequence, clone 72F05, translated in frame 1 in the form of SEQ ID NO:5. However, it was not known whether or not said SEQ ID NO:5 could have any biological function..

In view to obtain the instant non-adrenergic receptor including SEQ ID NO:1 or NO:13, plasmid DNA containing human clone designated 72F05 (EMBL accession n° z28655) (Auffray C. et al., 1995), including the corresponding coding sequence of SEQ ID NO:5 was obtained from Genethon, France and was used for preparing probes useful for hybridization assays.

900 bp probe (SEQ ID NO:3):

Cutting said plasmid DNA with restriction endonuclease EcoRI (New England Biolabs ref. 101 S) released a 0.9 kb insert corresponding to clone 72F05. This fragment was isolated using QiaEX II agarose gel extraction kit (Qiagen ref. 20021).

300 bp probe (SEQ ID NO:4):

1) Design of sens and anti-sens primers for PCR:

sens primer: S4 (SEQ ID NO:7)

anti-sens primer: S6 (SEQ ID NO:8).

2) PCR on clone 72F05:

Amplification was performed on 1 ng of plasmid DNA corresponding to clone 72F05, in the presence of the following reagents: each primer at 0.25 µM; 10% DMSO; 2.5 U of Taq polymerase (Promega); 0.25 mM of dNTP (dATP; dCTP; dGTP; dTTP); reaction buffer was supplied by Promega and supplemented with 1.5 mM $MgCl_2$.

PCR was performed on Perkin Elmer « Gene Amp PCR System 9600 »

using the following conditions:

4 min at 95°C		
---		
30 sec at 95°C	}	
30 sec at 48°C	}	30 cycles
30 sec at 72°C	}	
---		
4 min at 72°C		

Under these conditions, a 0.3 kb fragment corresponding to the published sequence of clone 72F05 was amplified. The fragment was isolated using QiaEXII agarose gel extraction kit (Qiagen ref. 20021).

- Radiolabeling of probes:

By random priming (Feinberg et al., 1983) 50 µCi of dATP α32P (ICN ref. 39010 X) were incorporated to radiolabel DNA fragments.

- Northern blot:

A human multiple tissue northern blot was purchased from Clontech (ref. 7765-1).

This blot ready to hybridize contained in each lane approximatively 2 µg of polyadenylated mRNA from 8 different human muscles (smooth and striated):

lanes 1-8 in order: human skeletal muscle, uterus (no endometrium), colon (no mucosa), small intestine, bladder, heart, stomach, and prostate (see figure 18A).

The membrane was hybridized following the suppliers instructions with labeled 300 bp probe (SEQ ID NO:4) (10^6 cpm/ml) during 24 hours.

Washes were carried out under different stringency:

1) low stringency: 2x SSC; 0.05% S.D.S. at room temperature.

Exposition of Amersham Hyperfilm MP at -80°C for 3 days using two intensifying screens showed three different fragments: 2 major bands are present in all samples; one at 3.4 kb and one at 3.8 kb. One fainter band, around 7 kb is found in all samples.

2) high stringency: 0.1 x SSC; 0.05% S.D.S. at 50°C; same exposition showed the same fragments in all samples.

The results are illustrated in figure 18 (same results with low or high stringency).

The visualized mRNAs correspond to SMBP transcripts. One explanation for the presence of 3 different transcripts could possibly be the utilization of alternative polyadenylation sites on SMBP gene (Intervening Sequences in Evolution and Development; E.M. Stone and R.J. Schwartz Oxford University Press 1990).

A similar analysis done with a variety of non muscular tissues (heart, brain, placenta, lung, liver, kidney and pancreas) confirmed these observations (figure 18B).

- Cloning of human cDNA:

A human skeletal muscle cDNA library was purchased from Clontech (ref. HL 300s; lot 32288). 500,000 clones were transferred to nylon membranes (Hybond N+; Amersham) and screened by hybridizing either with probe 300 bp (SEQ ID NO:4) or with probe 900 bp (SEQ ID NO:3).

Hybridization conditions were:

600 mM NaCl; 60 mM Na-citrate; 8 mM Tris-HCl pH 7,5; 50 mM Na-phosphate; 1% Ficoll; 1% polyvinylpyrrolidone; 1% bovine serum albumine; 40% formamide; 0.2% SDS; 50 µg/ml salmon sperm DNA.

Radiolabeled probe was added at 10^6 cpm/ml and incubated overnight at 42°C.

Final washes were at 50°C; 0.1 x SSC; 0.05% SDS for 1 hour.

11 positive clones were identified by repeated rounds of screening.

Insert sizes were analyzed by simultaneous cutting with the following restriction endonucleases: Xba I/Hind III and Xba I/Bam HI (New England Biolabs). These enzymes released cDNA inserts from the vector pcDNA I (Invitrogen). All clones were sequenced with T7 and SP6 primers from both ends and found to be overlapping.

The longest cDNA insert (clones n°24 and n°15) was about 1.7 kb and the smallest was about 0.65 kb (clone n°2).

Clone 24 was sequenced on both strands using T7 and SP6 primers and the following specific primers:

Plus strand primers:

S4: SEQ ID NO:7

S6: SEQ ID NO:8

S8: SEQ ID NO: 9

Minus strand primers:

S5: SEQ ID NO:10

S7: SEQ ID NO:11

S9: SEQ ID NO:12.

DNA sequencing data showed a continuous open reading frame (SEQ ID NO:2 or NO:14); translation into protein sequence (SEQ ID NO:1 or NO:13) showed several hydrophobic stretches (figure 23), suggesting that these regions are putative membrane spanning parts of the protein. The sequences corresponding to said hydrophobic stretches are highlighted (boxes) in figure 24.

SMBP appears to share structural homologies with members of a group of proteins described as « similar » to *Saccharomyces cerevisiae* EMP 70 protein precursor.

Figure 22 shows that:

- human myeloblastic cell line D87444 (Nagase T. et al., DNA Res., 1996, **3**, 321-329) is 30% homologous to SMBP,

- p76 protein (Schimmöller F. et al., accession number U81006) is 27% homologous to SMBP,

- the yeast endomembrane protein (Emp70) which is a precursor of a 24 kDa protein (Emp24) involved in intracellular vesicular trafficking (Schimmöller F. et al., EMBO J., 1995, **14**, 7, 1329-1339) is 23% homologous to SMBP,

- hMP70 (Chluba-de Tapia J. et al., Gene, 1997, 197, 195-204) is 28,5% homologous to SMBP whereas

- a protein from *Arabidopsis thaliana* (accession number U95973) is 51,2% homologous to SMDP.

The hydropathy plot of SMBP bears remarkable similarities to those of p76 protein, the myeloblast derived protein, hMP70 protein, *Arabidobsis* protein and Emp70 protein (see figure 23).

The affinity-labeled peptide sequence is located at the switch region between the hydrophobic N-terminal part of SMBP and the C-terminal hydrophobic stretch which contains the transmembrane regions.

The absence of N-glycosylation sites, the lack of homology with plasma membrane receptors and the similarity to intracellular proteins suggest that SMBP could indeed also be an intracellular membrane protein. SMBP appears to be expressed in many different tissues, and could therefore play a major role in normal cellular function. Since SMBP appears to be quite homologous to at least Emp70, involved in intracellular trafficking, i.e. ER via Golgi apparatus; this could also be a role for SMBP.

Example 4: Construction of a plasmid for the expression of Hu-SMBP.

For *in vitro* expression in mammalian cells, 1.7 kb cDNA insert of clone 24 was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). Simultaneous cutting by restriction endonuclease Xba I and Hind III (New England Biolabs) released the 1.7 kb insert from the pcDNA I vector (see example 3). The fragment was then blunt ended using Klenow fragment (Maniatis et al., Molecular Cloning, 2nd edition, 1, 5.40) and purified on 0.7% agarose gel using QiaEX II agarose gel extraction kit (Qiagen ref. 20021).

Vector pcDNA3 was cut in the multisite linker by Eco RV (New England Biolabs) and dephosphorylated using calf intestinal alkaline phosphatase (New England Biolabs). After heat inactivation of phosphatase, the vector and the insert were ligated using T4 DNA ligase (New England Biolabs). Subclone 3 was selected (designated as clone n°24.3). This plasmid contains at least SEQ ID NO:2.

Said recombinant plasmid may be transfected into mammalian cell lines for *in vitro* expression.

Example 5: Expression of SMBP in COS cells.

COS cells were transiently transfected with a vector containing the SMBP nucleotide sequence. The antibodies raised against a synthetic peptide (α8 antibodies) corresponding to the affinity-labeled fragment of rat SMBP were used for immunoprecipitation of proteins extracted from COS cells transfected with the human SMBP cDNA and labeled by I^{125}iodine using the chloramine T procedure. The precipitate was then redissolved and submitted to SDS-PAGE. A single protein with an apparent molecular weight of 45 kDa was identified after autoradiography (figure 25).

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As emerges from the foregoing, the invention is no way limited to those of its embodiments and modes if implementation and application which have just been described more explicitly; it encompasses, on the contrary, all variants which may occur to the specialist in the field, without departure from the scope or range of the present invention.